Western Blot protocol

1 Cell lysis and protein concentration check:

①Use RIPA buffer to lyse cells

For 6-well plates, 150 – 200 µl for each well;

For 100 mm plates, 500 -1000 µl for each plate

②After adding the RIPA buffer, leave the plate on ice for 30 min and transfer the supernatants to 1.5 ml tubes

③Spin the tubes at 13,000RPM for 10 mins, then transfer the supernatants to new tubes (be careful not to touch the pellets).

2 Check the protein concentrations with BCA assay

①Follow the kit' s protocol. In brief, right before use, mix the solution A and solution B at 1:50 ratio.

②Use a 96-well plate, take 200 µl mixed A and B into each well, do a replicate for each sample.

③Depend on the protein conc., take 1-5 µl to each well, gently shake the plates to mix the samples well;

④Leave the plate into a 37C incubator for 30 min, then check the absorbance at 570 nm

⑤For the first time, make a standard curve with BSA (1 mg/ml), add 0, 1 µl, 2µl, 4µl, 8 µl, 16 µl, totally 6 points. It should give a linear standard curve. Use Excel to get a formula, later on, use the formula to get the protein concentration. No need to do standard curve every time, which will save you time and doesn' t have any impact on your results.

⑥Having got the protein concentration, adjust the concentrations of your samples to the same by adding RIPA buffer and 4xSDS loading buffer. For instance, if you have 4 samples, 100 µl each, the concentrations are 1 µg/µl, 2µg/µl, 2.5 µg/µl, 3 µg/µl, you can do as follows:

Samples:	A	B	C	D
Conc.(µg/µl)	1	2	2.5	3
Amount of(µl) Samples	90	45	36	30
RIPA(µl)	0	45	54	60
4xSDS loading Buffer(µl)	30	30	30	30
Total(µl)	120	120	120	120

⑦By doing this, the final total protein concentrations of all the samples will be the same so that you can load the same volume next

RIPA buffer (Radio Immuno Precipitation Assay buffer)

150 mM sodium chloride

1.0% NP-40 or Triton X-100

0.5% sodium deoxycholate

0.1% SDS (sodium dodecyl sulphate)

50 mM Tris, pH 7.4

Add the proteinase inhibitor and phosphatase inhibitor right before use

one pill per 10 ml

3 Make SDS-PAGE gel：

①Depending on the size of your target proteins, you can choose the concentration of gel from 7.5% -20%. Usually, 10% is a good start. Make the gel accordingly.

The time for gel to polymerize is about 30 min. Too long will waste your time while too short won’ t give you an even gel. The APS solution is not stable, so make the fresh solution as least once a week. You will see that the gel won’ t polymerize if you choose to use the very old APS solution. When you try to save time or trouble not making fresh solution, you may end up spending more by going back to the beginning.

②Run gel at 120 V for 60 min (you need to adjust the time and voltage until the dye goes to the bottom).

③Transfer condition: 75 V for 75 min, add two pieces of blue ice to the transfer tank, which should be enough to cool the buffer.

④After transfer, wash briefly the blot with TBST, then incubate the blot in 5% milk in TBST for one hour, then wash the blot again three time with TBST, dilute the primary antibodies with 1% BSA in TBST, incubate at 4C for overnight (for most of antibodies, overnight will be good. For the antibodies like actin or tubulin, 1 hour at room temperature should be good enough). You can use either plastic bag or a box for the primary antibody incubation. For a box, 10 ml of diluted antibody should be enough. For a bag, use 2 to 5 ml depending on the size of your blot. Make sure to get rid of the air bubble as much as you can when using the plastic bags.

⑤Next day, transfer the diluted antibody to a 15 ml tube and keep it. In most cases, the diluted antibodies can be used repeatedly. Depending on the antibodies, the ones, such as actin and tubulin, can be used for 10 times. Most of them can be re-used for three times. You need to find out this information by your own experience. For a short time period, you can keep the diluted antibodies at 4C to avoid the repeated freezing and thawing.

⑥After removing the primary antibody, wash the blot with TBST, 3 times, 5 – 10 min each time. Then dilute the secondary antibody in 5% milk in TBST at 1:5000, incubate on an incubator for 1-2 hour, then wash the blot with TBST, 3 times, 5-10 min each time.

4 Blot development

We have three different kinds of Western blot substrates:

Supersignal west pico,

Supersignal West femto

Millipore - Immobilon Western Chemi HRP Substrate.